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TITLE: Modifiers of the Efficacy of Risk-Reducing Salpingo-Oophorectomy for the Prevention of Breast and Ovarian Cancer in Carriers of BRCA1 and BRCA2 Mutations

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**14. ABSTRACT**
The principle investigator was funded via a Physician-Scientist Training Award to participate in a comprehensive training plan to foster the transition to independent clinical breast cancer researcher. This plan included 1) conduct of a prospective study examining modifiers of the efficacy of risk-reducing salpingo-oophorectomy for the prevention of breast and ovarian cancer in carriers of BRCA mutations; and 2) participation in didactic coursework and structured training in research methodology, biostatistics, molecular biology, and ethics.

Progress from 5/1/2005 – 4/30/2006 includes: 1) Presentation of the first prospective data able to examine the efficacy of risk-reducing salpingo-oophorectomy for the prevention of BRCA-associated breast and gynecologic cancer when BRCA1 and BRCA2 mutation carriers are examined separately; 2) Publication of the first prospective data examining the risk of breast and ovarian cancer in BRCA-negative hereditary breast cancer families; and 3) Publication of a robust method for testing for BRCA1 and BRCA2 founder mutations in formalin-fixed paraffin-embedded tissue; 4) Continuation of formal training in genetic epidemiology laboratory methods, outcomes analysis, and conduct of clinical research; and 5) Receipt of an R03 award (1 R03 CA119265-01 to N.D.K.) to conduct a genetic epidemiologic, computational and structural analysis of BRCA2 variants of uncertain significance.

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## Introduction

The principle investigator was funded beginning on May 1, 2003 by the Department of Defense Breast Cancer Research Program via a Physician-Scientist Training Award (PTSA) to participate in a comprehensive training plan designed to assist the principal investigator in making the transition from junior faculty member to independent clinical breast cancer researcher. There were two chief components of the plan. The first component was the conduct of a prospective research study entitled, "Modifiers of the Efficacy of Risk-Reducing Salpingo-Oophorectomy for the Prevention of Breast and Ovarian Cancer in Carriers of *BRCA1* and *BRCA2* Mutations," under the direction and mentorship of Kenneth Offit, M.D., M.P.H. The second component of the comprehensive training plan was for the principal investigator to participate in didactic coursework and structured training in research methodology, biostatistics, methods of molecular biology, and ethics of clinical research. This progress report will summarize progress and accomplishments made as well as difficulties and challenges encountered during the third year of this award that ran from May 1, 2005 through April 30, 2006. This report will also summarize recent research findings that were presented at the June 2006 Meeting of the American Society of Clinical Oncology.

## 1) Progress on Research Project Component of Award

The principal investigator in concert with a multidisciplinary team at Memorial Sloan-Kettering Cancer Center (MSKCC) reported the first prospective evaluation of the role of salpingo-oophorectomy in reducing the risk of both breast cancers and *BRCA*-related gynecologic (ovarian, fallopian tube, and primary peritoneal) cancers in carriers of *BRCA1* and *BRCA2* mutations. In that study, we demonstrated that risk-reducing salpingo-oophorectomy (RRSO) is associated with a decreased combined incidence of breast and *BRCA*-related gynecologic cancer. While these results were encouraging, there were important limitations in that preliminary data that need to be addressed to allow better tailoring of risk reduction strategies for women at inherited risk secondary to a mutation in either *BRCA1* or *BRCA2*.

First, it is not at all clear that all women with *BRCA* mutations share the same cancer risks. The current study addresses the biologically plausible possibility that women with *BRCA2* mutations may not derive the same preventive benefit following oophorectomy as women with *BRCA1* mutations. Data pertaining to this issue may be important for the development of specific risk-reduction strategies for women with *BRCA* mutations. Second, it is also not clear that surgery will necessary improve *mortality* due to breast or ovarian cancer. Prospective information addressing the actual effect of RRSO on subsequent cancer-specific mortality is critically needed in order that women with *BRCA* mutations can make informed decisions regarding the risks and benefits of preventive surgery. Third, determining the specific risk reduction conferred by RRSO for the prevention of specific types of cancer is an important unanswered question for many women with *BRCA* mutations considering the procedure. The only data available on this issue at the time of the initiation of this award was retrospective with potential for substantial bias.

In order to address some of these issues, with the assistance of the PSTA, we have been conducting a prospective study to address the following three specific aims: #1) determine the degree of protection conferred by RRSO for the prevention of subsequent breast and *BRCA*-related gynecologic cancer in a) carriers of *BRCA1* mutations and b) carriers of *BRCA2* mutations; #2) determine the effect of RRSO on cancer-specific mortality in carriers of *BRCA1* and *BRCA2* mutations; and #3) determine the effect in carriers of *BRCA* mutations of RRSO on the incidence of a) subsequent breast cancer and b) subsequent *BRCA*-related gynecologic cancer.

The study plan is to ascertain women with a *BRCA1* or a *BRCA2* mutation, who have undergone genetic counseling at MSKCC, and who had not undergone bilateral oophorectomy prior to the time of receipt of genetic test results. Uptake of RRSO or use of ovarian surveillance is determined for study participants by

a combination of annual questionnaire, telephone contact, and medical record review. The time to cancer or time to cancer-specific mortality is analyzed for each of the specific aims using Kaplan-Meier analysis and a Cox proportion hazards model. Total planned accrual through April 30, 2007 is 452 participants with ovarian tissue at risk and 348 participants with both breast and ovarian tissue at risk, and accrual is continuing at a greater than expected rate.

In order to further increase our power, we have also initiated a collaboration with Dr. Timothy Rebbeck of the University of Pennsylvania and the Prevention and Observation of Surgical Endpoints (PROSE) study group. In this collaboration, we are combining our updated prospective follow-up data with data obtained from a similar prospective follow-up study being conducted at 10 North American and European centers. This collaboration has resulted in the ascertainment of a total 886 *BRCA* mutation carriers (597 with breast tissue at risk) in which a mean of 40 months of prospective follow-up is available. We have completed preliminary analysis on this combined cohort of the planned endpoints for specific aims #1 and #3 and have included a summary of these results in Section 3.

*Specific components of the statement of work for June 2005 – May 2006 relevant to the research component of the training award:*

a) June 2005 - Sept 2005: Preparation of manuscripts Based on data collected through the 2nd interim analysis.

   This component of the statement of work was conducted from June 2005 through January 2006 and resulted in the submission of an abstract addressing the planned endpoints of specific aims #1 and #3 to the 2006 Meeting of American Society of Clinical Oncology. Details of this abstract are described in section 3 and the actual abstract is attached in the appendix.

b) Sept 2005 – Jan 2006: Preparation of revised applications for continued support of PI's research on prevention of breast and gynecologic cancer in carriers of *BRCA* mutations.

   In September 2005, the principal investigator received an R03 award from the NCI Cancer Prevention Research Small Grant Program (1 R03 CA119265-01 to N.D.K.) to conduct a combined structural, computational and epidemiologic analysis of *BRCA2* missense mutations of uncertain clinical significance. Specific details of this project are included in Section 3 and abstract of the funded application is included in the appendix.

   In October 2005, the principal investigator submitted an R01 Application (1 R01 CA123164-01, Kauff ND - PI) to fund a "Prospective Cohort Study of Gynecologic Cancer Risk-Reduction Strategies in HNPCC" This is a cooperative group trial to be conducted through the Gynecologic Oncology Group to address gynecologic cancer screening and risk-reducing surgery in women with Hereditary Non-Polyposis Colon Cancer (HNPCC) syndrome. This protocol has been approved for further development by Protocol Committee of the Gynecologic Oncology Group as well as the CCOP program of NCI Division of Cancer Prevention. Initial scientific review occurred in March 2006 and a revised application will be submitted in November 2006.

   In February 2006, the principal investigator collaborated with investigators from the Abramson Cancer Center at University of Pennsylvania to submit an R01 application (Domchek S. – PI; Kauff ND – Subcontract PI) to assemble a multi-institutional cohort of *BRCA*-negative hereditary breast cancer kindreds to further address both cancer risks and efficacy of risk-reduction strategies in this important, but understudied, group of women.

c) April 2006 - May 2006 – 3rd Interim Data Analysis

This data analysis was completed as part of the preparation for the presentation of the data from the combined MSKCC/PROSE collaboration. The findings from this data analysis are summarized in Section 3 as noted above. A copy of the presentation is also included in the appendix.

**2) Progress of Didactic Training Component of Award**

Part of the time freed by the PSTA is also to be used by the Principal Investigator to participate in formal coursework and training in research methodology, biostatistics, methods of molecular biology, and ethics of clinical research. Specifics accomplishments relevant to this award are detailed below.

*Specific components of the statement of work for June 2005 – May 2006 relevant to the didactic and practical training component of the training award:*

a) June 2005 - May 2006: Participation in Weekly Meeting of the Diagnostic Molecular Genetics Laboratory at MSKCC.

Principal investigator was an active participant in these meetings with participation in laboratory based research projects as described below.

b) June 2005 - May 2006: Participation in Structured Laboratory Projects as Directed by Kenneth Offit, M.D., M.P.H.

During the period being reported on the principal investigator participated in two chief projects. The first of these was a project to validate a clinical test to evaluate founder *BRCA* mutations in paraffin embedded tissue. This is an ongoing project that builds upon work done during year 2 of the award. Details of this project are included in Section 3. A manuscript resulting from this work was recently published in the Journal of Medical Genetics. (Reprint attached in appendix) The second project was the continuation of a genetic epidemiologic, computational and structural analysis of *BRCA2* variants of uncertain significance. Preliminary data from this project was used to support an R03 application from the NCI Cancer Prevention Research Small Grant Program. This application was funded in September 2005. (1 R03 CA119265-01 to N.D.K.)

c) September 2005 – June 2006: Participation in Year 2 Modules of the Clinical Research Methodology Curriculum at MSKCC.

Due to change in the scheduling of the program, the principal investigator was unable to participate in the planned course modules of the curriculum. In order to substitute for the relevant experience, Kenneth Offit, MD, MPH, who is Chief of the Clinical Genetics Service at MSKCC, provided structured tutorials in the context of a weekly one on one clinical research meeting. Specific components of these tutorials included intensive training in Outcomes Analysis and Conduct of Clinical Investigations, which were two of the chief components of the Clinical Research Methodology Curriculum in which the principal investigator was scheduled to participate. Specific work products that resulted from these tutorials were: 1) The multi-center collaboration, which the principal investigator spearheaded, examining the impact of risk-reducing salpingo-oophorectomy for the prevention of *BRCA*-associated breast and gynecologic cancer described below; 2) the preparation of a subcontract on an R01 (Kauff ND – Subcontract PI) to further examine cancer risks and efficacy of risk-reduction strategies in *BRCA*-negative

hereditary breast cancer families; and 3) The preparation of an R01 Application (1 R01 CA123164-01, Kauff ND - PI) to fund a prospective cohort study to evaluate efficacy of risk-reducing hysterectomy for the prevention of HNPCC-associated gynecologic cancers. In the course of preparing this last application, the principal investigator's clinical research training was supplemented by extensive interaction with Roger Priore, Ph.D., a senior biostatistician with the Gynecologic Oncology Group, and members of the study steering committee which included a number of experienced senior clinical researchers including: Mark Greene, M.D., Chief, Clinical Genetics Branch, National Cancer Institute; David Mutch, M.D., Chief, Division of Gynecologic Oncology, Washington University in Saint Louis; Jeff Boyd, Director, Breast and Gynecology Research Laboratory, Memorial Sloan-Kettering Cancer Center; and Lari Wenzel, Ph.D., Chair, Quality of Life Committee, Gynecologic Oncology Group.

**3) Specific Research Findings Supported by This Award**

**A) Formed a multi-center collaboration to prospectively evaluate efficacy of risk-reducing salpingo-oophorectomy (RRSO) for the prevention of *BRCA*-associated breast and gynecologic cancer when carriers are stratified by mutation status.**

With the assistance of a Department of Defense Physician Scientist Training Award, the principal investigator has continued research on efficacy of RRSO for the prevention of breast and gynecologic cancers. Over the last year, we initiated collaborations with investigators from the University of Pennsylvania (Rebbeck TR, Domchek S) and the PROSE study consortium to create a multi-center consortium to answer questions regarding RRSO that could not be answered in the single institution setting. The first product of this collaboration was an abstract presented at the 2006 Meeting of the American Society of Clinical Oncology that provided the first prospectively determined estimates of efficacy of RRSO when mutation carriers are stratified by mutation status.

As part of this study, we assembled 886 women with documented *BRCA* mutations from eleven U.S. and European centers for which a mean of 40 months prospective follow-up was available. We were able to show that RRSO was profoundly protective against subsequent gynecologic cancers in carriers of mutation in both *BRCA1* (HR=0.13, 95% CI 0.04 – 0.46, p=0.003) and *BRCA2* (HR=0.00, 95% CI – Not estimable). We also showed that there may be a difference in the magnitude of protection conferred by RRSO against subsequent breast cancer between carriers of mutations in *BRCA1* (HR=0.61, 95% CI = 0.30 – 1.22, p=0.16) and *BRCA2* (HR=0.28, 95% CI = 0.08 – 0.92, p=0.036).

**B) Pilot Analysis of Risk of Ovarian Cancer in women from *BRCA*-negative hereditary breast cancer families**

Using time freed up by the PSTA, the PI continued his work evaluating cancer risk in *BRCA*-negative hereditary breast cancer families. As summarized last year, we evaluated the incidence of breast and ovarian cancer in 171 women from *BRCA*-negative hereditary breast cancer families who were prospectively followed for a mean of 3.6 years. The results of this evaluation were published in the Journal of the National Cancer Institute in September 2005. (Reprint attached in Appendix) Using this report as preliminary data, in concert with Susan Domchek, MD and Timothy Rebbeck, Ph.D. at University of Pennsylvania, we have submitted an R01 Application (Domchek s – PI, Kauff ND Subcontract –PI) to evaluate the cancer risks in and the efficacy of breast cancer risk-reduction strategies in *BRCA*-negative hereditary breast cancer families.

**C) Assisted in the development and validation of a reliable method for *BRCA1* and *BRCA2* founder mutation analysis in paraffin-embedded tissue**

A major limitation in counseling unaffected women from families with inherited breast and ovarian cancer is that a 'true-negative' interpretation of wild-type *BRCA* analysis of the proband can not be inferred in the absence of demonstration of a *BRCA* mutation segregating in the kindred. Documentation of familial *BRCA* mutations from paraffin-derived DNA of deceased patients has been limited due to reports of technical complications leading to lack of reproducibility of *BRCA* testing of archival material. In order to address this issue, the principal investigator in concert with a team from Kenneth Offit's lab, performed a blinded analysis of coded DNA samples extracted from paraffin embedded non-tumorous tissue from 161 women previously genotyped for the three Ashkenazi Jewish *BRCA* founder mutations from lymphocyte-derived DNA. Multiplex PCR on denaturing polyacrylamide gels was performed for the three founder mutations on DNA extracted from the paraffin embedded tissue. After disclosure of the sample codes, the results were compared with the original lymphocyte-derived DNA genotypes. Excluding one sample inevaluable due to poor quality of paraffin DNA, there was a 100% concordance of 160 genotypes derived from DNA from archival paraffin embedded tissue or peripheral lymphocytes. These results suggest that this method can reliably detect *BRCA* founder mutations in archival DNA derived from paraffin embedded tissue. These results will likely be useful in clinical settings to inform wild-type *BRCA* results of unaffected probands, leading to avoidance of unnecessary participation in risk-reduction strategies such as intensified surveillance or risk-reducing surgery. This work was recently published in Familial Cancer (Reprint Attached in Appendix)

**Key Research Accomplishments**

- Presented the first prospective data that has been powered to evaluate the efficacy of risk-reducing salpingo-oophorectomy for the prevention of *BRCA*-associated breast and gynecologic cancer when *BRCA1* and *BRCA2* mutation carriers are looked at separately. These results confirmed that RRSO is profoundly protective against subsequent breast and gynecologic cancers in carriers of mutations in both *BRCA1* and *BRCA2*. These results also suggested, however, that the magnitude of protection conferred against subsequent breast cancer differed between carriers of *BRCA1* and *BRCA2* mutations.

- Presented the first data providing evidence that women from *BRCA*-negative site-specific breast cancer families are not at increased risk of ovarian cancer. If confirmed, these results may allow women from *BRCA*-negative hereditary breast cancer data to avoid having to participate in ovarian cancer risk-reduction strategies.

- Participated in a research group that demonstrated a robust method of testing for the common *BRCA1* and *BRCA2* Ashkenazi founder mutation in formalin fixed paraffin embedded tissue. These results have the potential to inform the evaluation of Ashkenazi hereditary breast ovarian cancer families in which there was no living individual available to test.

**Reportable Outcomes**

- Presented data on impact of RRSO on *BRCA*-associated breast and gynecologic risk in carriers of mutations in *BRCA1* and *BRCA2* separately.

  **Kauff ND**, Domchek SM, Friebel TM, Lee JB, Roth R, Robson ME, Barakat RR, Norton L, Offit K, Rebbeck TR, and the PROSE Study Group. Multi-center prospective analysis of risk-reducing salpingo-oophorectomy to prevent *BRCA*-associated breast and ovarian cancer. Journal of Clinical Oncology, 2006 Vol 24, No. 18S, 2006: 1003

- Published data on ovarian cancer risk in *BRCA*-negative hereditary breast cancer families.

  **Kauff ND**, Mitra M, Robson ME, Hurley KE, Chuai S, Goldfrank D, Wadsworth E, Lee J, Cigler T, Borgen PI, Norton L, Barakat RR, Offit K. Risk of Ovarian Cancer in *BRCA1* and *BRCA2* Mutation Negative Hereditary Breast Cancer Families. Journal of the National Cancer Institute 2005; 97:1382-4.

- Published data on robust method of founder mutation testing in formalin-fixed paraffin embedded tissue.

  Adank MA, Brogi E, Bogomolniy F, Wadsworth EA, Lafaro KJ, Yee CJ, Kirchhoff T, Meijers-Heijboer EJ, **Kauff ND**, Boyd J, Offit K. Accuracy of *BRCA1* and *BRCA2* Founder Mutation Analysis in Formalin-Fixed and Paraffin-Embedded (FFPE) Tissue. Familial Cancer 2006 May 25; [Epub ahead of print].

**Conclusions**

With the support of the PTSA, the principle investigator is participating in a comprehensive training plan designed to assist him in making the transition from junior faculty member to independent clinical breast cancer researcher. Additionally, time freed by the PTSA has allowed the principal investigator to pursue several productive avenues of research addressing cancer risks in individuals who may be at inherited risk of breast and gynecologic cancer. It is anticipated that continued support from the PTSA will continue to further the principal investigator's development and ability to become an effective and highly productive clinical breast cancer researcher.

**Multi-center prospective analysis of risk-reducing salpingo-oophorectomy to prevent *BRCA*-associated breast and ovarian cancer**

N. D. Kauff, S. M. Domchek, T. M. Friebel, J. B. Lee, R. Roth, M. E. Robson, R. R. Barakat, L. Norton, K. Offit, T. R. Rebbeck, and the PROSE Study Group; Memorial Sloan-Kettering Cancer Center, New York, NY; University of Pennsylvania School of Medicine, Philadelphia, PA

**Background:** Our groups previously reported on the efficacy of risk-reducing salpingo-oophorectomy (RRSO) for the prevention of *BRCA*-associated breast and ovarian cancer. (Kauff ND, et al. NEJM 2002; Rebbeck TR, et al. NEJM 2002) Limitations of those reports included relatively short prospective follow-up and lack of power to analyze the protection of RRSO when participants were stratified by *BRCA1* vs. *BRCA2*. To address these limitations, we have pooled our updated datasets to provide robust estimates of the efficacy of RRSO. **Methods:** 886 women ≥ 30 years of age, with a deleterious mutation in *BRCA1* or *BRCA2* and ovaries in-situ at time of genetic test results, were enrolled on prospective follow-up studies at one of eleven centers from 11/1/1994 - 12/1/2004. Women chose to participate in either ovarian surveillance or undergo RRSO. Follow-up information was collected by questionnaire and medical record review. Follow-up time was counted from time of RRSO or from time of genetic test results for women who did not undergo RRSO. After excluding cancers diagnosed within the first 6 months of follow-up, the effect of RRSO on time to diagnosis of breast or *BRCA*-associated gynecologic cancer was analyzed using a Cox proportional-hazards model. **Results:** 561 (63%) participants underwent RRSO a median of 5 months after genetic test results. 15 occult ovarian or fallopian tube cancers were diagnosed at time of RRSO. During a mean 40 months follow-up, RRSO was associated with a 47% reduction in breast cancer risk and an 89% reduction in ovarian cancer risk (see Table). When the cohort was stratified by mutation status, RRSO was associated with a reduced risk of *BRCA1*-associated ovarian cancer and *BRCA2*-associated breast cancer. **Conclusions:** The results confirm that RRSO is highly protective against *BRCA*-associated breast and ovarian cancer. These results also generate the hypothesis that the protection conferred by RRSO against specific cancers may differ between carriers of *BRCA1* and *BRCA2* mutations.

Table 1. Hazard of Breast or Ovarian Cancer Following Risk-Reducing Salpingo-Oophorectomy

| | | Ovarian Cancer | | | | Breast Cancer | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Group | N | FU (Years) | Events | HR (95% CI) | N | FU (Years) | Events | HR (95% CI) |
| Total | RRSO | 546 | 3.4 | 3 | 0.11 (0.03-0.37) | 303 | 3.0 | 19 | 0.53 (0.30-0.97) |
| | No RRSO | 325 | 3.2 | 14 | | 294 | 2.8 | 28 | |
| *BRCA1* | RRSO | 352 | 3.4 | 3 | 0.13 (0.04-0.46) | 190 | 3.0 | 15 | 0.61 (0.30-1.22) |
| | No RRSO | 198 | 3.3 | 12 | | 178 | 2.8 | 19 | |
| *BRCA2* | RRSO | 194 | 3.2 | 0 | Not Estimable | 113 | 3.1 | 4 | 0.28 (0.08-0.92) |
| | No RRSO | 127 | 3.0 | 2 | | 116 | 2.7 | 9 | |

This work was partially supported by the Department of Defense Breast Cancer Research Program (DAMD17-03-1-0375 to N.D.K., DAMD-17-03-1-0619 to S.M.D.), the U.S. Public Health Service (R01-CA83855 and R01-CA102776 to TRR); the Koodish Fellowship Fund, the Lucius L. Littauer Foundation, the Frankel Foundation, QVC Network, the Fashion Footwear Association of New York, the Edward Spiegel Memorial Fund, the University of Pennsylvania Cancer Center, and the Prevention, Control and Population Research Program of Memorial Sloan-Kettering Cancer Center.

## Multi-Center Prospective Analysis of Risk-Reducing Salpingo-Oophorectomy to Prevent *BRCA*-Associated Breast and Ovarian Cancer

ND Kauff, SM Domchek, TM Friebel, JB Lee, R Roth, ME Robson, RR Barakat, L Norton, K Offit, TR Rebbeck, and the PROSE Study Group

Memorial Sloan-Kettering Cancer Center, New York, NY;
University of Pennsylvania School of Medicine, Philadelphia, PA

## Initial Studies Evaluating RRSO in Carriers of *BRCA* Mutations

- Kauff ND, et al. NEJM 2002; 346:1609-15
  - Prospective Cohort
  - 98 RRSO 72 Surveillance, Mean FU 24 months
    - Breast and Ovary: HR= 0.25 (95% CI 0.08 – 0.74)
- Rebbeck TR et al. NEJM 2002; 346: 1616-22
  - Case-Control/Cohort
  - 259 RRSO 292 Surveillance, Mean FU 8.5 years
    - Breast: HR = 0.47 (95% CI 0.29 – 0 .77)
    - Ovary: HR = 0.04 (95% CI 0.01 – 0. 16)

## Summary of Studies Examining Impact of RRSO in Carriers of *BRCA* Mutations

| | Hazard Ratio for GYN Cancer | Hazard Ratio for Breast Cancer |
|---|---|---|
| Kauff, et al. NEJM 2002 | 0.15 (0.02-1.31) | 0.32 (0.08-1.20) |
| Rebbeck, et al. NEJM 2002 | 0.04 (0.01-0.16) | 0.47 (0.29-0.77) |
| Rutter, et al. JNCI 2003 | 0.29 (0.12-0.73) | -- |
| Eisen, et al. JCO 2005 | -- | 0.44 (0.29-0.66) |
| Domchek et al. Lancet Oncol 2006 | 0.11 (0.03-0.47) | 0.36 (0.20-0.67) |

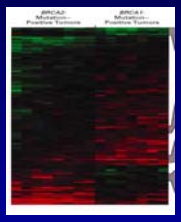
## Limitations of Prior Reports

- Short follow-up of prospective series
- Potential for ascertainment and survival biases in the case-control series
- Inclusion of prevalent cancers in all but one series
- All series to date – Inadequate power to examine differences in outcome between carriers of *BRCA1* and *BRCA2* mutations

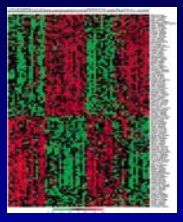
## Mutations in *BRCA1* and *BRCA2* Cause Distinct Cancer Susceptibility Syndromes

- Breast Cancer
  - *BRCA1*: 10-24% ER positive
  - *BRCA2*: 65-79% ER positive
- Ovarian Cancer
  - *BRCA1*: 34-46% risk (to age 70)
  - *BRCA2*: 10-27% risk (to age 70)

## *BRCA1* and *BRCA2* Associated Tumors Have Different Gene Expression Profiles



Breast
Hedenfalk I, et al. NEJM 2002



Ovary
Jazaeri A, et al. JNCI 2002

## Goals

- To separately determine the efficacy of RRSO for the prevention of subsequent *BRCA*-associated breast and gynecologic cancer in *BRCA1* and *BRCA2* mutation carriers.

## Methods

- We updated and combined follow-up data from two large prospective cohorts of *BRCA* mutation carriers
  - Memorial Sloan-Kettering Cancer Center (MSKCC)
  - Prevention and Observation of Surgical Endpoints (PROSE) Study Group

## Eligibility Criteria

- Enrolled on IRB approved prospective follow-up studies
- Deleterious mutation in *BRCA1* or *BRCA2*
- Ovarian tissue at risk at time of genetic test results
- ≥ 30 years of age at start of follow-up
- Prospectively followed for ≥ 6 months

## Exclusion Criteria

- Prior bilateral oophorectomy
- Prior ovarian, fallopian tube or peritoneal cancer
- Stage IV disease prior to results
- Diagnosis of an occult gynecologic malignancy at time of RRSO
- Diagnosis of a "prevalent" cancer in the first 6 months of follow-up

## Study Cohort

1080 mutation carriers identified

- Exclusions:
  - 166 < 6 months of follow-up
  - 4 both *BRCA1* and *BRCA2* mutations
  - 15 occult gynecologic cancers at time of RRSO
  - 20 patients with prevalent breast cancer
  - 4 patients with prevalent gynecologic cancer
- 871 Patients in study cohort

## Statistical Analysis

- Follow-up calculated from date of genetic test results (no RRSO cohort) or date of RRSO (surgery cohort)
- Follow-up through date of last contact, date of post-results cancer, date of death.

## Statistical Analysis
### Breast Cancer Endpoint

- Patients with prior bilateral breast cancer or prior bilateral mastectomy were excluded
- For patients with a prior unilateral breast cancer, only contralateral breast was considered to be at risk.
- Participants were censored for the breast cancer outcome at time of post-results cancer, bilateral mastectomy, date of last follow-up or date of death

## Hazard Ratio Analysis

- A Cox proportional-hazard model adjusted for demographic variables significantly different between the RRSO and no RRSO cohorts was used to determine the Hazard Ratios (HR) for subsequent breast and *BRCA*-associated gynecologic cancer following RRSO.

## Study Cohort

- Baylor Univ (1)
- Creighton (52)
- Dana Farber (107)
- Fox Chase (27)
- Georgetown (76)
- MSKCC (354)
- Royal Marsden (35)
- St. Mary's (54)
- UC Irvine (34)
- Univ of Penn (105)
- Yale (26)

## Patient Characteristics

| | RRSO N=546 | No RRSO N=325 | P Value |
|---|---|---|---|
| Follow-up (mo.) | 40.4 | 38.4 | |
| Age (yrs.) | 47.1 | 43.3 | <0.001 |
| *BRCA1* (%) | 64 | 61 | 0.31 |
| Hx of Breast Ca (%) | 59 | 46 | <0.001 |
| Hx of OC Use (%) | 70 | 71 | 0.94 |
| Hx of HRT Use (%) | 11 | 7 | 0.05 |
| Parous (%) | 83 | 74 | 0.001 |

## Patient Characteristics:
### Cohort with breast tissue at risk (N=597)

| | RRSO N=303 | No RRSO N=294 | P Value |
|---|---|---|---|
| Follow-up (mo.) | 36.4 | 33.2 | |
| Age (yrs.) | 47.7 | 42.8 | <0.001 |
| *BRCA1* (%) | 63 | 61 | 0.62 |
| Hx of Breast Ca (%) | 47 | 37 | 0.013 |
| Hx of OC Use (%) | 71 | 72 | 0.99 |
| Hx of HRT Use (%) | 15 | 7 | 0.003 |
| Parous (%) | 81 | 75 | 0.06 |

## *BRCA*-associated Gynecologic Cancers Diagnosed during Follow-up

During 40 months mean follow-up:

- *BRCA1*
  - RRSO (N=352): 3 primary peritoneal cancers
  - No RRSO (N=198): 10 ovarian, 1 fallopian tube, 1 peritoneal
- *BRCA2*
  - RRSO (N=194): No cancers
  - No RRSO (N=127): 2 ovarian cancers

## *BRCA1*-associated Breast Cancers Diagnosed during Follow-up

During 35 months mean follow-up:

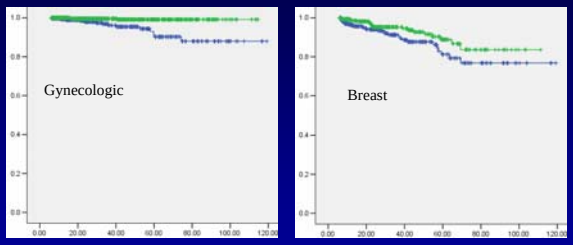
- RRSO (N=190)
  - 13 Invasive, 2 DCIS
    - Of invasive cancers: 1 ER+, 12 ER-
- No RRSO (N=178)
  - 13 Invasive, 3 DCIS, 3 Unknown
    - Of invasive cancers: 3 ER+, 9 ER-, 1 unknown

## *BRCA2*-associated Breast Cancers Diagnosed during Follow-up

During 35 months mean follow-up:

- RRSO (N=113)
  - 3 Invasive, 1 DCIS
    - Of invasive cancers: 1 ER+, 2 ER-
- No RRSO (N=116)
  - 5 Invasive, 4 DCIS
    - Of invasive cancers: 4 ER+, 1 ER-

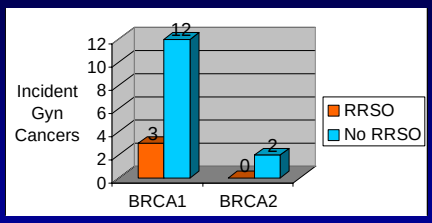
## Incident Cancers (*BRCA1* and *BRCA2* Combined)




Gynecologic: HR = 0.11 (95% CI 0.03 – 0.37), p<0.001

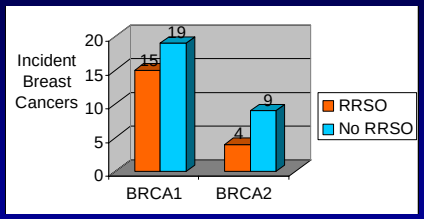
Breast: HR = 0.53 (95% CI 0.30 – 0.97), p=0.038

## HR for GYN Cancer Following RRSO (Stratified by Mutation Type)




- *BRCA1*: HR = 0.13 (95% CI 0.04 – 0.46), p=0.002
- *BRCA2*: HR = 0.00 (95% CI - Not Estimable)

## HR for Breast Cancer Following RRSO (Stratified by Mutation Type)




- *BRCA1*: HR = 0.61 (95% CI 0.30 – 1.22), p=0.16
- *BRCA2*: HR = 0.28 (95% CI 0.08 – 0.92), p=0.036

## Impact of RRSO on ER-positive vs. ER-negative Breast Cancer (*BRCA1/BRCA2* Combined – Adjusted for Mutation Type)

| | | ER-positive | | | ER-Negative | | |
|---|---|---|---|---|---|---|---|
| | N | Events | HR (95% CI) | P | Events | HR (95% CI) | P |
| RRSO | 300 | 2 | 0.22 (0.05-1.09) | 0.06 | 14 | 1.27 (0.54-2.99) | 0.58 |
| No RRSO | 283 | 7 | | | 10 | | |

## Conclusions

- In the largest prospective follow up study to date, RRSO significantly reduced the risk of:
  - *BRCA*-associated gynecologic cancer in both *BRCA1* and *BRCA2* mutation carriers
  - Breast cancer in *BRCA2* mutation carriers
- Magnitude of breast cancer risk reduction in *BRCA1* mutation carriers, while not statistically significant, is consistent with estimates from prior studies

## Conclusions

- There appear to be differences in the magnitude of protection against breast cancer conferred by RRSO (perhaps caused by differences in the breast cancer phenotype) between *BRCA1* and *BRCA2*.
- Future studies will need to stratify for mutation type as risk-reduction strategies may have different effects in these two related but distinct cancer susceptibility syndromes.

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**Structural, Computational and Epidemiologic Analyses of *BRCA2* Missense Mutations**

**1 R03 CA119265-01**

**Principal Investigator – Noah D. Kauff, M.D.**

A major limitation of genetic testing for *BRCA* mutations using sequence-based approaches is that missense mutations of uncertain clinical significance are frequently identified. Despite the identification of such missense mutations in 13-17% of individual who undergone genetic testing, there is no information regarding the biologic significance of these changes in the majority of these cases to assist in guiding clinical management. In order to address this issue, we propose to conduct a combined structural, computational and epidemiologic analysis of *BRCA2* missense mutations. Briefly, we plan to develop and refine a computational protocol to incorporate structural modeling and protein superfamily analysis to predict the biologic significance of specific *BRCA2* missense mutations. We will then use this computational protocol to gain insight into the functional importance of *BRCA2* missense mutations that have been frequently reported to the Breast Cancer Information Core database. Missense mutations predicted by the computational analysis to likely be functionally significant will then be analyzed in association and co-segregation studies in an attempt to further elucidate the functional significance of these mutations. The combined approach used in this proposal will build upon identified strengths at our institution in Structural and Computational Biology, Genetic Epidemiology, and Clinical Genetics. This combined approach we believe will allow us to develop what we hope to be a more powerful and biologically relevant method of analyzing *BRCA2* variants of uncertain significance, which if confirmed, will be directly and immediately translatable to individuals and families with these mutations.

# Risk of Ovarian Cancer in BRCA1 and BRCA2 Mutation-Negative Hereditary Breast Cancer Families

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**Women from site-specific hereditary breast cancer families who carry a BRCA1 or BRCA2 mutation are at increased risk for ovarian cancer. It is less clear, however, whether individuals from hereditary breast cancer families who do not carry such a mutation are also at increased ovarian cancer risk. To determine whether women from BRCA mutation–negative hereditary breast cancer families are at increased risk for ovarian cancer, 199 probands from BRCA mutation–negative, site-specific breast cancer kindreds who consented to prospective follow-up at the time of genetic testing were identified. The incidence of new breast and ovarian cancers in probands and their families since receipt of their genetic test results was determined by questionnaire. The expected number of cancers and standardized incidence ratios (SIRs) were determined from age-specific cancer incidence rates from the Surveillance, Epidemiology, and End Results (SEER) program by using the method of Byar. All statistical tests were two-sided. During 2534 women-years of follow-up in 165 kindreds, 19 new cases of breast cancer were diagnosed, whereas only 6.07 were expected (SIR = 3.13, 95% confidence interval [CI] = 1.88 to 4.89; *P*<.001), and one case of ovarian cancer was diagnosed, whereas only 0.66 was expected (SIR = 1.52, 95% CI = 0.02 to 8.46; *P* = .48). These results suggest that women from BRCA mutation–negative, site-specific breast cancer families are not at increased risk for ovarian cancer. [J Natl Cancer Inst 2005;97:1382–4]**

Women with deleterious mutations in the BRCA1 or BRCA2 genes have a 9- to 36-fold increased risk of breast cancer and a 6- to 61-fold increased risk of ovarian cancer compared with general population rates *(1)*. Because of the incomplete sensitivity of current methods to detect mutations in BRCA1 and BRCA2 *(2–4)* and because of reports of breast and ovarian cancer kindreds that do not show linkage to either BRCA1 or BRCA2 *(2,5)*, women from mutation-negative hereditary breast cancer families may be recommended to participate in ovarian cancer risk-reduction strategies, including intensive screening and/or risk-reducing surgery *(6–8)*. However, such strategies may subject women whose ovarian cancer risks are not clear to inconvenience, expense, anxiety, invasive follow-up, and the sequelae of surgical menopause as a result of oophorectomy. To address this issue, we conducted a prospective study of women from BRCA mutation–negative, site-specific hereditary breast cancer kindreds to evaluate their risk of subsequent ovarian cancer.

Records of 1745 patients of the Clinical Genetics Service at Memorial Sloan-Kettering Cancer Center (MSKCC) who underwent testing for BRCA1 and BRCA2 mutations from August 1, 1996, through July 31, 2002, and who provided written informed consent for prospective follow-up on one of two institutional review board–approved studies were reviewed. This cohort represented 95.8% of all individuals who underwent BRCA mutation testing at MSKCC during the study period. All BRCA mutation–negative, site-specific breast cancer kindreds with a living female proband were identified. We included probands if 1) the kindred had at least three cases of breast cancer in the same lineage, 2) one of the breast cancers in a kindred was diagnosed when the patient was younger than age 50 years, 3) no ovarian cancer was present anywhere in the lineage, and 4) BRCA mutation screening did not detect a deleterious or unclassified missense mutation in the proband's BRCA1 or BRCA2 gene. If the proband reported her heritage to be exclusively Ashkenazi, testing negative for the three Ashkenazi founder mutations was sufficient for study inclusion because testing for just these mutations has been shown to identify approximately 95% of detectable BRCA mutations in such individuals *(9,10)*. We defined a proband as the youngest living individual with breast cancer in the kindred who had personally undergone BRCA mutation testing.

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*See* "Notes" following "References."

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**Table 1.** Participant demographics

| Demographic | Value |
|---|---|
| Total No. of participants | 165 |
| Mean age, y (range) | 51.6 (25–77) |
| Personal history of breast cancer, No. (%) | 128 (77) |
| Mean age at diagnosis of breast cancer in probands, y (range) | 48.5 (24–74) |
| Mean No. of breast cancers in kindred (range) | 4.14 (3–9) |
| No. Ashkenazi Jewish (%) | 110 (67) |

We used this proband definition because BRCA mutation testing in these individuals, as opposed to kindred members who presented first but were unaffected or diagnosed with breast cancer at later age, would be most likely to provide informative results. If a family had no member who had both been diagnosed with breast cancer and had undergone genetic testing, the proband was defined as the first unaffected individual in the kindred who underwent testing. All probands were sent a detailed follow-up questionnaire to obtain clinical follow-up information and detailed information on new cancers that they and their first-degree and second-degree relatives might have developed. Probands who did not respond to the mailed questionnaire were contacted by telephone and asked to provide follow-up information via a structured interview.

For each kindred, the number of women-years at risk for the proband and her relatives was the difference between the date follow-up information was provided and the date genetic testing results were transmitted to the proband. Expected cancer incidence for probands and for all first-degree and second-degree relatives in the lineage at risk older than 18 years at the time that results were transmitted to the proband was based on age-specific Surveillance, Epidemiology, and End Results (SEER) rates from 1973 through 2001 in 5-year age groups, beginning with age 15 years and ending with age 85 years or older *(11)*. If the age of a relative in the same generation as the proband was not known precisely, we assumed it to be that of the proband. If the relative was in the earlier or subsequent generation, we assumed her age to be 25 years older or younger than the age of the proband, respectively. The observed women-years of risk were then multiplied by expected cancer incidence obtained from the SEER database to estimate the total expected number of cancers. Standardized incidence ratios (SIRs) were determined by calculating the ratio of observed to expected numbers of cancers. The 95% confidence intervals (CIs) were calculated by using the method of Byar *(12)*. The chi-square test was used to calculate *P* values. All statistical tests were two-sided.

Two hundred and seven living female probands meeting the study criteria were identified and sent a study questionnaire. Eight questionnaires were returned because of incorrect contact information. Of the remaining 199 probands, 165 (83%) completed the study questionnaire either by mail or telephone interview. Demographics of the study participants are presented in Table 1. Study participants were less likely to be of Ashkenazi heritage than nonresponders (67% vs. 88%, $P = .01$) There were no other statistically significant differences in any demographic criteria between study participants and nonresponders.

During a mean follow-up of 40.6 months (range = 15.3–82.4 months), seven of 165 probands and 12 of their 583 first-degree or second-degree female relatives had a new diagnosis of breast cancer, compared with 6.07 diagnoses that were expected among these 748 individuals (SIR = 3.13, 95% CI = 1.88 to 4.89; $P<.001$). The 19 cases of breast cancers were diagnosed in 17 different kindreds a mean of 2.2 years after the proband received genetic test results. The mean age at diagnosis was 54.9 years. No proband and only one first-degree relative had ovarian cancer diagnosed during the 2534 women-years of follow-up, compared with 0.66 that were expected in this cohort (SIR = 1.52, 95% CI = 0.02 to 8.46; $P = .48$). This case of ovarian cancer was diagnosed in a 64-year-old sister of a proband, 4 years after the proband received genetic test results. Table 2 shows observed versus expected numbers of breast and ovarian cancers when the cohort is stratified by degree of relation.

Previous studies in ungenotyped women with a personal and family history of breast cancer have suggested that these women are at increased risk of developing ovarian cancer compared with the general population *(13,14)*. Because the percentage of women in these studies with a deleterious BRCA1 or BRCA2 mutation is unknown, the incremental risk for ovarian cancer in women from BRCA mutation–negative hereditary breast cancer families is unclear. Additionally, because current BRCA mutation detection techniques are only 63%–85% sensitive *(2,15)* and because linkage studies have suggested that 10%–12% of hereditary breast cancer families with one case of ovarian cancer do not segregate a BRCA1 or BRCA2 mutation *(2)*, many cancer genetic services suggest that women in BRCA mutation–negative hereditary breast cancer families consider participation in ovarian cancer risk-reduction

**Table 2.** Standardized incidence ratios (SIRs) for breast and ovarian cancer with 95% confidence intervals (CIs)*

| Cancer | Cohort | No. | No. observed cancers | No. expected cancers | SIR (95% CI) | *P* value |
|---|---|---|---|---|---|---|
| Breast | Overall | 748 | 19 | 6.07 | 3.13 (1.88 to 4.89) | <.001 |
| | Probands | 165 | 7 | 1.43 | 4.90 (1.96 to 10.11) | <.001 |
| | First-degree relatives | 321 | 8 | 2.46 | 3.25 (1.40 to 6.40) | .004 |
| | Second-degree relatives | 262 | 4 | 2.18 | 1.83 (0.49 to 4.69) | .17 |
| Ovary | Overall | 748 | 1 | 0.66 | 1.52 (0.02 to 8.46) | .48 |
| | Probands | 165 | 0 | 0.14 | 0.00 (NA to 25.60) | .45 |
| | First-degree relatives | 321 | 1 | 0.26 | 3.88 (0.05 to 21.60) | .22 |
| | Second-degree relatives | 262 | 0 | 0.26 | 0.00 (NA to 14.30) | .31 |

*Confidence intervals were calculated by using the method of Byar *(12)*. *P* values were calculated by the chi-square test. All statistical tests were two-sided. NA = not applicable (lower limits for 95% confidence interval cannot be calculated using the method of Byar when the SIR is zero).

strategies. Our results, if confirmed, may allow this approach to be modified.

There are several possible sources of bias in this study. First, it is possible that some Ashkenazi Jewish probands may have had an undetected nonfounder BRCA mutation. Second, in a subset of kindreds, the genotyped proband was unaffected. The inclusion of such unaffected probands could result in the ascertainment of kindreds with BRCA mutations that did not segregate in the proband. Finally, because only one individual was genotyped in the majority of kindreds, phenocopies (i.e., patients with sporadic cancer in the background of an inherited predisposition) may have also resulted in undetected BRCA mutations in a fraction of kindreds. In all three of these cases, the result would be a bias toward the null hypothesis with more ovarian cancers being observed than expected.

Although these results suggest that no increased risk of ovarian cancer is associated with site-specific hereditary breast cancer kindreds with a BRCA mutation–negative status, caution is advised before women from these families are counseled not to participate in ovarian cancer risk-reduction strategies because there are several important limitations of this study. Two-thirds of the women in the cohort were Ashkenazi Jewish, and it is possible that BRCA mutation testing in this group more effectively excludes the possibility of a deleterious mutation than in non-Ashkenazi populations. Additionally, our study was powered to detect a 3.5- to 4-fold increase in ovarian cancer risk compared with that of the general population. Detection of a smaller (2.5- to 3.0-fold) increase in ovarian cancer risk in a study with a comparable design would require 3800–7600 women-years of follow-up compared with the 2534 women-years of follow-up in this study. The level of risk detected in such a study would be comparable to that of an individual with a first-degree relative with ovarian cancer; currently such individuals are not recommended to participate in ovarian cancer risk-reduction strategies outside of clinical trials *(16)*.

Despite these limitations, the current study provides the first prospective evidence, to our knowledge, that women from BRCA mutation–negative, site-specific hereditary breast cancer families may not be at statistically significantly increased risk of subsequent ovarian cancer. If these results are confirmed by other studies, it may allow ovarian cancer risk-reduction strategies to be tailored to women from site-specific breast cancer kindreds.

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## Notes


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ORIGINAL PAPER

# Accuracy of *BRCA1* and *BRCA2* founder mutation analysis in formalin-fixed and paraffin-embedded (FFPE) tissue

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**Abstract**
*Background* A major limitation in counseling unaffected women from families with inherited breast and ovarian cancer is that a ''true-negative'' interpretation of wild type *BRCA* analysis of the proband cannot be inferred in the absence of demonstration of a *BRCA* mutation segregating in the kindred. Documentation of familial *BRCA* mutations from paraffin-derived DNA of deceased patients has been limited due to reports of technical complications leading to lack of reproducibility of *BRCA* testing of archival material.
*Methods* DNA was extracted from formalin-fixed paraffin-embedded (FFPE) morphologically normal tissue of 161 blinded, coded samples from women previously genotyped for the three Ashkenazi Jewish *BRCA* founder mutations from lymphocyte-derived DNA. Multiplex PCR followed by denaturing polyacrylamide gel electrophoresis was performed for the three founder mutations to determine if analysis on FFPE tissue could produce results concordant with those of the lymphocyte-derived DNA.
*Results* After disclosure of the sample codes, the results were compared with the original lymphocyte-derived DNA genotypes. Excluding one sample unevaluable due to PCR failure, there was 100% concordance of 160 genotypes (120 mutation samples) derived from DNA from archival FFPE tissue compared to peripheral lymphocytes.
*Conclusions* The method described reliably detected *BRCA* founder mutations in archival DNA derived from FFPE tissue. These results suggests that this technique may be useful in clinical settings to inform wild type *BRCA* results of unaffected probands, leading to avoidance of unnecessary intensified surveillance or risk-reducing surgery. With further validation this approach can also be applied to other populations where founder mutations are observed.

**Keywords** *BRCA* · Breast and ovarian cancer · FFPE · Founder mutation · Multiplex PCR

**Abbreviations**

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| FFPE | Formalin-fixed and paraffin-embedded |
| PCR | Polymerase chain reaction |
| del | Deletion |
| ins | Insertion |
| MSKCC | Memorial Sloan-Kettering Cancer Center |

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## Introduction

Germline mutations in the breast cancer genes *BRCA1* and *BRCA2* are associated with over 80% of dominantly inherited breast/ovarian cancer families. In many ethnic groups there is an elevated frequency of recurrent mutations in the *BRCA* genes due to common ancestral origins and endogamy (founder mutations). Most of the *BRCA* founder mutations cause frameshifts due to a deletion (del) or an insertion (ins).

In the Ashkenazi Jewish population, the founder mutations *BRCA1*185delAG*, *BRCA1*5382insC* and the *BRCA2*6174delT* are commonly observed [1–3]. Together these three mutations account for approximately 95% of detectable *BRCA* mutations found in dominantly inherited early onset breast and ovarian cancer families of Ashkenazi Jewish ancestry [4, 5]. Founder mutations are also present in parts of the Netherlands, in the French-Canadian population, as well as in Iceland, Hungary, Sweden, Norway, Russia, Poland and in certain Asian countries [6–9].

Ideally, *BRCA* testing should commence with an ovarian cancer case or the youngest affected woman with breast cancer in a hereditary breast and ovarian cancer kindred, in order to maximize the likelihood of detecting a mutation, and to serve as a point of reference for other family members [10]. However, it is not uncommon to encounter women from dominantly inherited breast (and ovarian) cancer kindreds in which all affected individuals are deceased. In this setting, the finding of wild type sequence in selected *BRCA* founder alleles is of limited clinical utility, as these women may have other *BRCA* mutations, or may have mutations in other breast cancer susceptibility genes [4, 11]. Most often, women with such ''uninformative negative'' results are recommend to participate in a program of tailored surveillance because of the residual risk for breast and ovarian cancer. In such settings prophylactic mastectomy may still be considered because of the significant proportion of hereditary site specific breast cancer that is not linked to *BRCA1* or *BRCA2*, as well as the possibility that the predisposition to cancer is caused by either a non-founder or non-detectable *BRCA* mutation [11]. Less commonly, oophorectomy may also be considered in this setting, although the risk of ovarian cancer in *BRCA* wild type kindreds does not appear to be elevated [12]. Analysis of DNA derived from archival pathology material of deceased affected individuals in such kindreds offers the promise of clarifying these ''uninformative negative'' interpretations for unaffected probands, mitigating the need for intensified surveillance and/or risk-reducing surgery.

Unfortunately, there have been reports of technical difficulties in amplifying DNA from paraffin-embedded tissue using different techniques [13–16]. DNA degradation from aging, fixatives, type of Taq DNA polymerase and length of PCR product have been reported as being responsible [13, 17, 18]. In this study we employed a rapid and reliable acrylamide gel-based method to detect Ashkenazi Jewish *BRCA* founder mutations in archival FFPE tissue, which can be generalized to any population in which such ancestral mutations are observed.

## Materials

Five 10 μm thick unstained sections of morphologically normal formalin-fixed paraffin-embedded tissue were obtained from 161 blocks derived from 158 different women from 148 separate Ashkenazi Jewish kindreds. These sections were ascertained as part of an Institutional Review Board approved protocol. The preserved tissue blocks were dated from 1980 through 2004 and were kept at the pathology department of Memorial Sloan-Kettering Cancer Center. All tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE).

Kindreds were ascertained by the Clinical Genetics Service from 1995 to 2005. The only ascertainment criterion was that samples were derived from women who had lymphocyte DNA genotyping, which revealed mutant or wild type sequence (as controls) for any of the three founder mutations. In 109 kindreds (121 mutation carriers) a known founder mutation was detected in lymphocyte-derived DNA, and in 39 kindreds (40 controls) no founder mutation was detected in lymphocyte-derived DNA.

Morphologically normal tissue sections of 40 *BRCA2*6174delT*, 24 *BRCA1*5382insC*, 57 *BRCA1*185delAG* mutation carrier samples and 40 samples of non-heterozygotes were collected and delivered anonymously coded to the laboratory for DNA analysis (Table 1).

For each mutation, samples were grouped based on age of archival sample. One group consisted of samples biopsied before 1994 and another group consisted of samples biopsied after 1994. This grouping was performed to determine if age of archival tissue or changes in methods of tissue fixation would affect the reliability and reproducibility of genotyping. The formalin-fixed paraffin-embedded tissue from 25 collected samples was at least 10 years old (before 1994) (Table 1). The oldest sample was biopsied and preserved 25 years (1980) before DNA extraction and genotyping was performed.

**Table 1** Samples for analysis

| | Lymphocyte-derived DNA | FFPE tissue-derived DNA | |
|---|---|---|---|
| | | < 1994 | > 1994 |
| *BRCA1*185delAG* | 57 | 14 | 43 |
| *BRCA1*5382insC* | 24 | 3 | 21 |
| *BRCA2*6174delT* | 40 | 6 | 34* |
| Wild type | 40 | 2 | 38 |
| Total | 161 | 25 | 136* |

Samples of the three Ashkenazi founder mutations tested in lymphocyte-derived DNA by direct sequencing and tested in formalin-fixed and paraffin-embedded (FFPE) tissue divided by age of blocks. *One of the 161 FFPE tissue samples was excluded (0.6%) precluding adequate genotyping. DNA from this sample was derived from breast tissue and was preserved after 1994. The comparative lymphocyte-derived DNA from this case had shown a *BRCA2*6174delT* mutation

Morphologically normal tissue was derived for DNA-extraction from different tissue types, predominantly breast (74 out of 161) and ovarian (49 out of 161) tissue (Table 2). Often, samples were only small biopsies, for example, endometrial curettage material.

## Methods

### DNA extraction

Five 10 μm thick unstained sections of morphologically normal FFPE tissue for each sample were cut with a disposable blade and placed in 1.5 ml tubes.

The tissue in each tube was deparaffinized by the addition of 1.0 ml of octane (Fisher Scientific), vortexed at maximum speed for at least 20 s, adding 75 μl of methanol (Fisher Scientific), and vortexed 20 s. Samples were then centrifuged for 10 min at room temperature at 14,000 rpm and the supernatant was removed by pipetting using filter tips. The tissue pellet was air-dried and soaked in 100 μl of Cell Lysis Solution (Gentra Systems). Each sample was homogenized with sterile RNAse-free, disposable microcentrifuge pellet pestles (Fisher Scientific) followed by the addition of 200 μl of Cell Lysis Solution. Depending on the volume of the tissue, 30 or 60 μl of Recombinant PCR Grade Proteinase K (Roche Applied Science) was added and the tissue was digested by incubating in a waterbath at 55°C overnight. After cooling the samples to room temperature, 110 μl of Protein Precipitation Solution (Gentra Systems) was added to each tube and vortexed vigorously. Tubes were placed on ice for 10 min and centrifuged at 14,000 rpm for 10 min. The supernatant above the pellet was transferred to 1.5 ml tubes and 500 μl (approximately 1:1) isopropanol (Fisher Scientific) was added. After the addition of 1 μl Glycogen (20 μg/ml, Roche Applied Science) each tube was inverted and centrifuged at 14,000 rpm for 5 min to pellet the DNA. The supernatants were removed and the DNA pellets were washed with 70% ethanol, centrifuged at 14,000 rpm for 1 min and air-dried after ethanol removal.

Based on the pellet size 20 or 50 μl of DNA Hydration Solution (Gentra Systems) was added and each tube incubated at 65°C for 1 h. The DNA concentration was measured by spectrophotometry and then the DNA samples were stored at −20°C.

**Table 2** Overview of FFPE tissue types

| Tissue type | Number of samples |
|---|---|
| Breast/nipple | 74 |
| Ovary/fallopian tube | 49 |
| Endometrium/cervix | 15 |
| Skin | 8 |
| Lymph nodes | 7 |
| Gastrointestinal | 4 |
| Peritoneal adhesions | 3 |
| Muscle | 1 |
| Total | 161 |

Morphologically normal tissue was derived for DNA extraction. Often samples were only small biopsies

### DNA amplification and mutation analysis

The method of mutation screening utilized in this study is based on the original acrylamide gel approach used to describe the Ashkenazi founder mutations [19]. By multiplexing this technique, the size difference of the three mutant fragments is compared to wild type DNA. Such multiplexing allows detection of all three founder mutations simultaneously [20]. The primer sequences (three pairs) used for the Ashkenazi founder mutations are given in Table 3.

In brief, per sample, 10 μM of each forward primer was individually end-labeled in a reaction with 0.75 $\mu$Ci [$\gamma$-33P] adenosine triphosphate per sample, 1× Forward Reaction Buffer (Invitrogen) and 0.1 U T4 kinase (Invitrogen). Radiolabeled primers were then combined in a 10 μl multiplex PCR reaction consisting of 50 ng DNA, 0.2 mM of each dNTPs (Promega), 0.8 μM of each forward and reverse primer (Genosys), 10× PCR Buffer (Applied Biosystems), and 0.0625 U Ampli Taq Gold DNA Polymerase (Applied Biosystems). Amplification was then performed with a 10 min 95°C hot start, 35 cycles of 20 s 95°C, 20 s 55°C and 30 s 72°C each, followed by a 7 min extension at 72°C and ending with a 4°C hold. The PCR products were diluted 1:1 (10 μl) in denaturing loading buffer (95% formamide, 10 mM EDTA, 0.02% xylene cyanol and

**Table 3** Primer sequences

The forward (F) and reverse (R) primer sequences and PCR product sizes in base pairs (bp) for the Ashkenazi Jewish founder mutations

| Primers | Nucleotide sequence | Product size (bp) |
|---|---|---|
| *BRCA1* 185 del AG F | 5′ TCT GCT CTT CGC GTT GAA GAA 3′ | 90 |
| *BRCA1* 185 del AG R | 5′ CAC TCT TGT GCT GAC TTA CCA 3′ | |
| *BRCA1* 5382 ins C F | 5′ GAG GAG ATG TGG TCA ATG GAA 3′ | 80 |
| *BRCA1* 5382 ins C R | 5′ AGG GAG CTT TAC CTT TCT GTC 3′ | |
| *BRCA2* 6174 del T F | 5′ GGG AAG CTT CAT AAG TCA GTC 3′ | 97 |
| *BRCA2* 6174 del T R | 5′ TTT GTA ATG AAG CAT CTG ATA CC 3′ | |

0.02% bromophenol blue), heated at 95°C for 5 min and placed on ice before loaded. Products were visualized by running on a 6% denaturing polyacrylamide gel containing 7 M Urea and run in 1× TBE buffer for approximately 3 h at 80 W. Gels were dried at 80°C for 1 h under vacuum. Dried gels were exposed for at least 1 h on a PhosphorImager screen and visualized with Image-QuaNT software; only 4 h were needed to produce interpretable genotyping results. Gels were then exposed to film for at least 48 h and developed. All mutant genotypes were confirmed by repeat analysis.

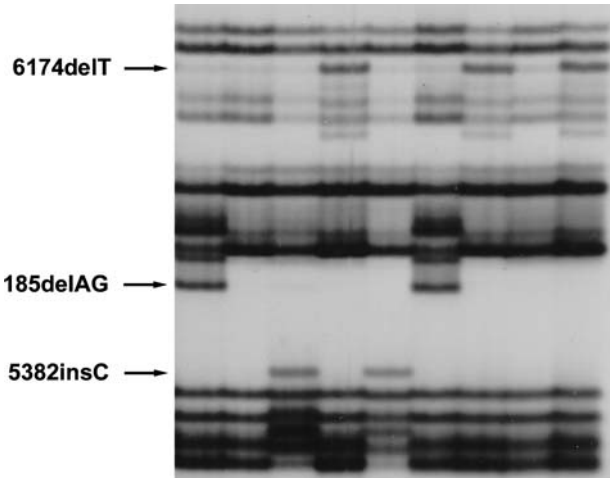


**Fig. 1** Denaturing polyacrylamide gel with founder mutation samples. PCR products for the three *BRCA* Ashkenazi founder mutations are shown simultaneously for each sample. Samples with a mutation show a distinct shifted band in comparison to the wild type DNA product on denaturing gels due to the size difference of the DNA fragment. In this gel, three 6174delT, two 185delAG and two 5382insC (one positive control) and two wild type samples are shown

## Results

The laboratory investigators were blinded to the lymphocyte-derived DNA genotyping results. Results of the archival DNA analysis were sent to the study coordinator. The sample codes were disclosed and the study coordinator compared the results from the coded samples with the original lymphocyte DNA results. One sample was excluded because of PCR failure (0.6%) precluding adequate genotyping. DNA from this sample was derived from breast tissue and was preserved after 1994. For the remaining 160 samples, there was 100% concordance between DNA samples derived from archival FFPE tissue and fresh lymphocytes (Table 1). There were no false negatives or false positives.

The age of the tissue blocks did not interfere with the results. All mutant genotypes were confirmed by repeat analysis; all samples with a mutation showed a very clear distinct band specific for that mutation due to size difference of the fragment and showing the same result as the concordant positive control on the same gel (Fig. 1).

## Discussion

Previously it has been reported that DNA degradation and suitability for PCR-based analysis of DNA derived from paraffin-embedded tissue is dependent on length of fixation, fixatives and reagents utilized. Different methods have been employed to detect small genetic alterations in DNA derived from paraffin-embedded tissue for genotyping of the tumor suppressor gene *p53* [15, 16, 21–23].

Only two publications performed a comparative, blinded study of testing *BRCA1* and *BRCA2* mutations on DNA derived from paraffin-embedded tissue, however, neither used a radioactive multiplex PCR method, and both studies used a small number of samples [6, 13]. Kuperstein et al. tested 30 samples derived from paraffin-embedded tumor tissue for the Ashkenazi Jewish and French-Canadian founder mutations and also found a concordance of 100% using a fluorescence-based multiplex PCR method. Bernstein et al. performed a multicenter study were they sequenced 12 DNA samples derived from paraffin-embedded tissue, but only obtained a 45–55% rate of correct identification of frameshift (deletion and insertion) mutations. This group reported that this rate was dependent on the age of the blocks being used.

Utilizing conventional sequence-based genotyping, we and another group have also noted inconsistencies in *BRCA* genotyping based on paraffin-derived DNA (Ellis NA and Bale A, personal communication).

In this study 120 samples with either one of the three *BRCA* Ashkenazi founder mutations including 25 samples over 10 years old were tested. No difference was found in the quality and reproducibility of the results between old and new samples. This multiplex PCR method described in this report is particularly suitable for screening for *BRCA1* or *BRCA2* mutations in the Ashkenazim, where three founder mutations appears to account for approximately 95% of all detectable mutations in this population [4, 5]. This method may also be usable in other geographic and ethnic groups where deleterious founder mutations accounts for a substantial fraction of hereditary breast and ovarian cancer linked to *BRCA1* or *BRCA2* [6]. However, such ''single amplicon'' based approaches for founder mutation detection can not substitute for full sequence analysis, since non-founder *BRCA1* and *BRCA*2 mutations may still occur in these genetic isolates.

The success of our multiplex PCR method can mainly be attributed to the small size of the amplicons. The PCR primers used for each of the three *BRCA* founder mutation yields PCR products under 100 bp. Since DNA quality from archival paraffin-embedded tissue may be suboptimal for the amplification of long fragments due to degradation, it is critical to minimize the length of the PCR products while still allowing for mutation detection. The use of radioactivity in our multiplex PCR method allows for increased sensitivity in the detection of product when small amounts of DNA are available. Additionally, the use of AmpliTaq Gold DNA Polymerase (Perkin–Elmer) with a hot start versus AmpliTaq DNA Polymerase resulted in increased specificity in the PCR reaction resulting in less non-specific products.

The 100% sensitivity of the genotyping approach used here validates its potential clinical role. Paraffin-based *BRCA* testing of archival material may have a significant clinical impact. For example, at MSKCC we have identified as many as 1,000 unaffected women from high risk breast and ovarian cancer families, who have been shown not to have one of the three common Ashkenazi founder mutations, but for whom no affected living relative was available for testing. Based on the strength of the family history, many of the individuals in this group are recommended to participate in both breast and ovarian risk-reducing strategies because of the possible increased risk of cancer caused by other germline genetic changes that were not excluded by the founder mutation testing. The majority of individuals in this group have deceased family members whose DNA could possibly be retrieved for archival DNA testing.

The advantages of establishing a ''true negative'' *BRCA* wild type result in these individuals are multifold. By decreasing risk estimates to a subset of these individuals and their families, exposure to risks of intensified surveillance and risk-reducing surgery can be substantially mitigated [24].

Certain barriers remain however before widespread testing of archival paraffin-derived DNA can be achieved. Importantly, insurance reimbursement for this testing is not assured at present. At least one US carrier (Aetna) covers the cost of *BRCA* testing for an affected relative of an unaffected proband, if that proband carries the insurance policy. It is unclear if such a policy will apply to deceased relatives, and if other insurance plans will follow suit. In addition, many pathology departments do not yet view paraffin archives as DNA repositories and may discard them over time.

Finally, psychological and practical considerations may limit the motivation and ability of women to pursue DNA testing of archival material from deceased relatives. Such material, legally, is under the custody of the executor of the estate of the deceased, and not the next-of-kin (who may not always be the same person). This may lead to emotional barriers precluding discussion of the process of taking legal custody of the archival material [25]. The psychological and emotional strains of entering into these discussions also cannot be taken for granted, and will require psychosocial supports as part of the pre-test counseling process.

However, despite these caveats, the availability of DNA testing for *BRCA* mutations on paraffin material marks another important advance for germline genetic testing. While underutilized at present, microsatellite testing of archival material has held the potential of *increasing* the probability of successful identification of candidates for hereditary non-polyposis colon cancer testing [26]. In contrast, utilizing a denaturing acrylamide gel-based approached, archival DNA testing for *BRCA* founder mutations offers the promise of *decreasing* the intensity of cancer screening and prevention recommendations for a subset of women with uninformative negative results who seek guidance regarding their hereditary cancer risk.

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